ATTACHMENT B

REMARKS

By this amendment, Applicants have amended the claims in a manner which overcomes all prior rejections and now places this application in condition for allowance. In particular, the claims have been amended so as to incorporate the subject matter of Claim 2 into Claim 1, namely the fact that the isolated antibody recognizing CNA19 is also capable of recognizing *S. epidermidis*, an unexpected beneficial result as shown herein and in the accompanying Declaration of Dr. Magnus Hook, Ph.D. Similar amendments have been made to Claim 23 which is now in condition for allowance for the same reasons as discussed herein. Finally, other minor amendments have been made in that Claim 26 is now properly dependent on Claim 23 which is what was originally intended, and new claims 33 and 34 replace canceled claims 24 and 25, and these claims are properly dependent on Claim 23 as well. Applicants submit that in light of the Amendments and submission herewith, the present application has been placed ion condition for allowance for the reasons as set forth below.

In the Official Action, the Examiner maintained obviousness-type double patenting rejections with regard to co-pending serial number 09/813,820 and U.S. Pat. No. 6,288,214. In addition, the Examiner maintained rejections under 35 U.S.C. §103 on the basis of U.S. Pat. No. 6,288,214 and WO 97/43314. Since all of these patent references are equivalents of the same original application, their disclosures are essentially the same, and Applicants' arguments apply to all of these references equally. In short, for reasons as set forth herein and in the attached Declaration of Dr. Magnus Hook, Ph.D., the prior references relating to the collagen binding proteins of *S.*

aureus do **not** disclose or suggest the present claims which are directed to an isolated *S. aureus* CNA19 antibody which has the unexpected and beneficial property of being able to cross-react with *S. epidermidis*.

In particular, as shown in the attached Declaration of Dr. Magnus Hook, Ph.D., the present claims relate in particular to an isolated antibody which not only recognizes the CNA19 region from *S. aureus*, it is also cross-reactive to *S. epidermidis* in a manner not possible prior to the present invention. As shown in the declaration, the cross-reactivity of the CNA19 antibody to *S .epidermidis* was very unexpected for a number of reasons. First, there are major differences between *S. aureus* and *S. epidermidis,* in terms of bacterial type, structure, and the different nature of the proteins and polysaccharides expressed therein, and thus it would not be expected that an antibody recognizing CNA19 of *S. aureus* would recognize epitopes from *S. epidermidis* as well. Moreover, *S. aureus* are coagulase-positive staphylococcal bacteria, and *S. epidermidis* are coagulase-negative staphylococcal bacteria, a fundamental and distinct difference between bacterial types. Further, many proteins and polysaccharides expressed in *S. aureus* are not expressed in *S. epidermidis*, and vice versa. Not surprisingly, in the common case, antibodies raised against *S. aureus* proteins do not commonly cross-react with *S. epidermidis*. See Hook Dec., ¶ 3.

More importantly, the present inventive group also discovered that the collagen-binding protein in *S. epidermidis* that appears to be recognized by antibodies to CNA19 is the GehD Lipase enzyme, and as indicated above, this enzyme has a structure which is radically different from that of the CNA protein. In particular, the GehD recognized by the antibody to CNA19 has a circular dichroism spectra that differs significantly from

that of CNA, and it does not have the conserved LPXTG C-terminal motif commonly present in CDNA proteins. Accordingly, because of the huge structural differences in the collagen binding proteins, which are similar to the many other differences normally found between *S. aureus* and *S. epidermidis* proteins, it was totally unexpected that an antibody which recognized CNA19 of *S. aureus* would also be able to recognize *S. epidermidis*. As a result of this unexpected property, cross-reactive CNA19 antibodies have the unexpected beneficial ability to recognize both *S. aureus* **and** *S. epidermidis* and thus can be useful in providing compositions that can be used against a much wider range of bacterial pathogens at one time.

Finally, in the Official Action, the Examiner stated that the antibodies disclosed in the cited patent references disclosed antibodies which "are cross-reactive to S. epidermidis." As shown in the attached Declaration, and as can be confirmed by a review of the cited references, no such antibodies are disclosed or suggested in those references. Accordingly, the claim that these references disclose antibodies cross-reactive to S. epidermidis" is totally inaccurate and has no basis in fact. Moreover, there is also no basis for the Examiner's argument that an antibody to a whole protein or a larger region of a protein automatically recognizes a lesser included region, and indeed this is contradicted in the evidence provided in U.S. Pat. No. 6,288,214 cited by the Examiner which shows that the antibody to the whole CNA protein and the antibody which recognized the specific region M55 were different antibodies and indeed exhibited different properties. In fact, if the two would have been considered the same, or if one was considered inherent in the other, than the U.S. Pat. No. 6,288,214 would not have

issued since it claims antibodies to the M55 region (SEQ ID NO: 6) and **not** antibodies to the whole CNA protein.

Simply put, the discovery of the antibody to CNA19 which is cross-reactive to both *S. aureus* and *S. epidermidis* was a new discovery that was unexpected in light of the prior work in this field and the knowledge of the great differences between *S. aureus* and *S. epidermidis*, and the present invention will provide benefits in terms of protection against both *S. aureus* and *S. epidermidis* in a manner not previously possible for a single composition. Accordingly, the rejections of the Examiner on the basis of the prior patent family including Ser. No. 09/813,820, U.S. Pat. No. 6,288,214 and WO 97/43314, insofar as applied to the claims as amended, are respectfully traversed and should be withdrawn.

In the Official Action, there were additional rejections on the basis of the Patti et al. 1992 and 1995 Articles, but the Examiner herself pointed out that these articles did not relate to Claim 2, and that Claim 2 was not rejected on the basis of these articles (see Official Action at page 9). Accordingly, in light of the fact that the subject matter of Claim 2 (cross-reactivity) has now been incorporated into independent Claims 1 and 23, these claims are acknowledged to be patentable over the cited Patti references, and any remaining rejections have also been traversed.

In light of the amendments and arguments as set forth above, as well as the Declaration appended hereto, Applicants submit that the present application overcomes all prior rejections and has been placed in condition for allowance. Such action is earnestly solicited.

END REMARKS

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. MAGNUS HOOK, PH.D.		
	Application #	09/810,428
	Confirmation #	6490
	Filing Date	19 March 2001
	First Inventor	HOOK et al.
	Art Unit	1645
	Examiner	Baskar
	Docket #	P06668US03/BAS

I, Dr. Magnus Hook, Ph.D., declare and state as follows:

1. I am one of the inventors of the above-identified patent application, and I am currently a faculty member of Texas A&M University Health Science Center Institute of Bioscience and Technology. I am also an adjunct professor in the Department of Cell Biology at Baylor College of Medicine, in the Department of Microbiology at the University of Texas Health Science Center at Houston and the Department of Molecular Genetics at the University of Texas M.D. Anderson Cancer Center. In addition, I am an inventor or co-inventor of numerous US Patents, particularly those involving bacterial extracellular matrix binding proteins and other isolated bacterial surface proteins, including US Patent Nos. including U.S. Pat. No. 6,703,025 for multicomponent vaccines derived from extracellular matrix proteins, U.S. Pat. No. 6,288,214 for Collagen Binding Protein Compositions and Methods of Use, U.S. Pat. No. 6,680,195, for Extracellular matrix-binding proteins from Staphylococcus aureus, U.S. Pat. No. 6,685,943, Fibronectin binding protein compositions and methods of use, and U.S. Pat. No. 6,692,739, Staphylococcal immunotherapeutics via donor selection and donor stimulation, and further have also authored or co-authored numerous journal articles in this field. I am thus well familiar with the subject matter of the present invention.

2. The present invention relates in particular to an isolated antibody which not only recognizes the CNA19 region from *S. aureus*, it is also cross-reactive to *S. epidermidis* in a manner not possible prior to the present invention. In fact, the cross-reactivity of the CNA19 antibody to *S .epidermidis* was very unexpected since there are major differences between the *S. aureus* and *S. epidermidis* proteins, in terms of amino acid sequence, secondary structure, predicted tertiary structure and mechanism of bacterial surface association. Thus it was totally unexpected that an antibody recognizing CNA19 of *S. aureus* would recognize epitopes from *S. epidermidis* as well.

3. From the available genome information the surface proteins on *S. aureus* and *S. epidermidis* are very different. Accordingly, in the common case, antibodies raised against *S. aureus* proteins do not commonly cross-react with *S. epidermidis*. One of many articles which have evidenced the differences in the surface proteins between *S. aureus* and *S. epidermidis* is Espersen et al., Infection and Immunity 49(3):700-708 (September 1985). A copy of this article is attached hereto as Appendix A.

4. Further, recent studies by my inventive group have shown that the collagen-binding protein in *S. epidermidis* that appears to be recognized by antibodies to CNA19 is the GehD Lipase enzyme which indeed has a structure which is radically different from that of the CNA protein. As shown in the attached article, Bowden et al., J. Biol. Chem. 277(45):43017-43023 (November 8, 2002, attached hereto as Appendix B), the GehD recognized by the antibody to CNA19 has far different properties than the

CNA protein, including the fact that the mature GehD circular dichroism spectra differs significantly from that of CNA, and it does not have the conserved LPXTG C-terminal motif commonly present in CDNA proteins.

5. Accordingly, because of the huge structural differences in the collagen binding proteins, which are similar to the many other differences normally found between *S. aureus* and *S. epidermidis* proteins, it was totally unexpected that an antibody which recognized CNA19 of *S. aureus* would also be able to recognize *S. epidermidis*. As a result of this unexpected property, cross-reactive CNA19 antibodies have the unexpected beneficial ability to recognize both *S. aureus* **and** *S. epidermidis* and thus can be useful in providing compositions that can be used against a much wider range of bacterial pathogens at one time.

6. Previously, my inventive group has worked extensively with the collagen binding CNA protein from *S. aureus*, and this work is reflected by our previous patent references, including WO 97/43314 and U.S. Pat. No. 6,288,214. However, these references do not disclose or suggest a CNA19 antibody which is cross-reactive to both *S. aureus* and *S. epidermidis*. I have reviewed the Official Action in the above application, and it is clear that the Examiner's claim that these references "disclose antibodies cross-reactive to S. epidermidis" is totally inaccurate and has no basis in fact. Simply put, the discovery of the antibody to CNA19 which is cross-reactive to both *S. aureus* and *S. epidermidis* was a new discovery that was unexpected in light of our prior

work and will provide benefits in terms of protection against both *S. aureus* and *S. epidermidis* in a manner not previously possible for a single composition.

I hereby state that all statements made herein based on my own personal knowledge are true and correct and that all statements based on my information and belief are true and correct to the best of my knowledge, and further that all of these statements have been made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

8/23/04
Date

Dr. Magnus Hook, Ph.D.

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Isolation of *Staphylococcus aureus* Clumping Factor

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Immunochemically identical components were isolated from water-soluble phases of five *Staphylococcus aureus* strains by affinity chromatography on fibrinogen-linked Sepharose 4B. The elution was performed with 1 M $MgCl_2$. The component could be isolated from sonicated preparations of whole cells, cell walls, and extracellular products of *S. aureus* but not from sonicated preparations of staphylococcal L-forms or from *Staphylococcus epidermidis*. Investigations of the eluted component by immunoelectrophoresis and Western blot analysis by use of different polyspecific antibodies to *S. aureus* raised in rabbits revealed only one immunoprecipitate or one band. By means of gel filtration on Sepharose CL 6B and sodium dodecyl sulfate-polyacrylamide gel electrophoresis a molecular mass of 420,000 and 360,000 was found, respectively. Chemical analysis showed a carbohydrate content of about 20% by weight. By crossed immunoelectrophoresis the isolated component was demonstrated to bind to human fibrinogen. The finding that this purified component inhibited the fibrinogen-induced clumping of staphylococci strongly suggests that the component is the *S. aureus* clumping factor.

A wide range of bacterial species have receptors on their cell surfaces for different mammalian plasma proteins. The most well known receptor is cell wall protein A (28) from *Staphylococcus aureus*, which binds human immunoglobulin G (IgG) through its Fc-part (17). Protein A has also been shown to have affinity for other human immunoglobulins (21) as well as immunoglobulins from other animal species (19, 37). Recently, it has been demonstrated that many other bacteria have protein A-like structures on their cell surfaces (7, 35). Although staphylococcal protein A has been isolated (27) and characterized (2, 44) and has been shown to be highly useful in different immunological techniques (18), none of the immunoglobulin receptors from other bacteria have yet been isolated. Only a few receptors for other plasma proteins on bacterial cell walls have been characterized. Human fibrinogen binds to the M-protein of streptococci (29), human fibronectin seems to bind to the lipoteichoic acid of the streptococcal cell wall (43), and recently a fibronectin-binding protein from *S. aureus* has been isolated (14).

Binding of fibrinogen to a component on the *S. aureus* cell wall is known to result in the agglutination of whole staphylococci. The component on *S. aureus* has been designated the clumping factor. In 1908 Much had already observed that some staphylococci clumped in the presence of plasma (39). The phenomenon was later believed to result from the enzyme staphylocoagulase which together with factor II (prothrombin) forms an enzymatic active complex resulting in the conversion of fibrinogen to fibrin (25). Coagulase was believed to exist in two forms, free coagulase and bound coagulase (6). However, Duthie has demonstrated that clumping factors differ from free coagulase in both mechanisms of action and in antigenic properties (10, 11). Few attempts have been made to isolate this staphylococcal clumping factor. The purification methods have been based on phenole extraction, acetone precipitation, or acid extraction (4, 31, 46).

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In this report, we describe the isolation of a fibrinogen-binding component from *S. aureus* by affinity chromatography on fibrinogen-linked Sepharose 4B. The results indicate that this component might be the staphylococcal clumping factor.

MATERIALS AND METHODS

Culture media. Truche medium containing peptone (40 g/liter; Orthana, Copenhagen, Denmark) with NaCl (0.086 M) and glucose (0.01 M) (pH 7.5) was used. Truche agar plates contained Noble agar (15 g/liter; Difco Laboratories, Detroit, Mich.). Hyperton agar plates with methicillin were prepared as described for truche agar plates but contained, in addition, 10^5 μg of methicillin per liter (Lucopenin; Lundbeck, Copenhagen, Denmark), $MgSO_4$ (0.016 M), NaCl (0.85 M), and a 0.05 volume of human serum with inactivated complement. Human serum was obtained from blood pooled from five normal, consenting persons. The complement inactivation was achieved by incubation of the serum at 56°C for 1 h.

Bacterial strains and culture conditions. All bacterial strains were kindly provided by K. Rosendal, Statens Seruminstitut, Copenhagen, Denmark. *S. aureus* E1369, a protein A-deficient strain, was isolated from blood cultures (41). *S. aureus* E2371, E2476, E1346, and E1430 have also been isolated from blood cultures and represent the four phage groups of *S. aureus* (14). All five strains were able to clump in the presence of purified fibrinogen (13). *Staphylococcus epidermidis* A1271/76, A1388/76, A1394/76, and A1389/76 have been characterized previously (15). None of these strains was able to clump in the presence of purified fibrinogen (13). All strains were stored freeze-dried, and when reconstituted, they were cultivated on truche agar for 18 h at 37°C. The bacteria were scraped off the plates and washed with 0.154 M NaCl (saline). The bacteria were separated by centrifugation at 6,000 × *g* for 10 min at 4°C. The washing procedure was repeated three times.

Preparation of extracellular products of *S. aureus*. Truche medium (0.8 liter) was inoculated with bacteria from several colonies of *S. aureus* and incubated at 37°C for 18 h. After

APPENDIX A

centrifugation twice at 6,000 × *g* for 10 min at 4°C, the supernatant was filtered through a Millipore filter (0.22-μm pore size; Millipore Corp., Molsheim, France), dialyzed against distilled water for 24 h, and concentrated to 0.025 volume by use of collodion bags (Sartorius, Göttingen, Federal Republic of Germany). Extracellular products were prepared from all five *S. aureus* strains. The protein concentrations were estimated to be 4.4 to 8.7 g/liter by absorbance at 280 nm and by assuming that $\varepsilon = 10\ M^{-1}\ cm^{-1}$ (40).

Preparation of wall-defective forms of *S. aureus* E1369. Wall-defective forms (L-forms) of strain E1369 were induced by methicillin by a modification of the method of Hamburger and Carleton (20). Strain E1369 was cultivated on hypertonic truche agar containing methicillin for 4 weeks at 37°C. The colonies were scraped off the plates and washed with 0.85 M NaCl followed by centrifugation at 12,000 × *g* for 10 min. Washing was repeated five times. By Gram staining, only gram-negative bacterial forms were seen.

Preparation of whole, heat-killed *S. aureus* E1369. *S. aureus* E1369 was suspended in saline and adjusted to a concentration of 6×10^{12} CFU per liter by measuring the absorbance at 650 nm. The concentration was confirmed by viable counts by a pour-plate method. The bacteria were heat killed at 100°C for 5 min.

Preparation of *S. aureus* cell walls. Undigested cell walls were prepared from all five *S. aureus* strains by a modification of the method of Yoshida et al. (50). Whole, washed bacterial cells were suspended in distilled water to a concentration of 400 g (wet weight) per liter. The cells were broken after freezing by three passages through an X-press (D-25; AB Biox, Nacka, Sweden). Whole, unbroken bacteria were removed by centrifugation twice at 3,000 × *g* for 15 min at 4°C. Cell walls were harvested from the final supernatant by centrifugation at 10,000 × *g* for 25 min at 4°C. The cell walls were washed six times with distilled water followed by centrifugation at 10,000 × *g* for 25 min at 4°C and lyophilized. From 20 g of whole bacteria, 40 to 110 mg of cell walls was obtained. Evaluation of the cell wall preparations by electron microscopy of negatively stained preparations was kindly performed by Jens Blom, Statens Seruminstitut (26). Electron microscopy showed the typical picture of cell walls, mainly composed of empty shells with few whole cells and no debris present.

Preparation of bacterial antigens by sonication. After sonication of whole bacteria (all strains) water-soluble supernatants were prepared as described previously (14). Briefly, whole, washed bacterial cells, cell walls, or L-forms were suspended in distilled water to a concentration of 250 g/liter and disintegrated by sonication at 20,000 Hz/s which was repeated three times for 5 min (whole cells and cell walls) or for 30 s (L-forms) with a Rapidis 300 19-mm probe with 9.5-mm tip cooled with ice water. The disintegrated material was centrifugated at 48,000 × *g* for 1 h at 4°C, and the supernatants were filtered through a Millipore filter (0.22-μm pore size). The sonicated preparations were stored at −20°C. Protein concentrations were estimated to be 3.2 to 8.0 g/liter by determining the absorbance at 280 nm.

Polyspecific antibodies against staphylococci. Five rabbits were immunized by one intravenous injection per week of 6×10^9 CFU of whole, heat-killed *S. aureus* E1369 and were bled after 6 weeks. Four groups of five rabbits each were immunized intracutaneously for 16 months by the immunization and bleeding schedule described by Harboe and Ingild (22). The antigens used for immunization of the different groups were sonicated preparations of *S. aureus* E1369 (5.2 g/liter); equal volumes of a mixture of sonicated preparations of *S. aureus* E2371, E2476, E1346, and E1430 (final concentration, 4.2 g/liter); equal volumes of a mixture of sonicated preparations of the four *S. epidermidis* strains (6.1 g/liter); and equal amounts of a mixture of cell walls from all five *S. aureus* strains (10 g/liter). Each rabbit received 0.1 ml of antigen in 0.1 ml of incomplete Freund adjuvant per injection. Immunoglobulins from the rabbit sera were isolated and concentrated (22). The final preparations contained 19.2 to 31.0 g of protein per liter.

Fibrinogen. Human fibrinogen was obtained from IMCO, Stockholm, Sweden. Contaminating fibronectin was removed by affinity chromatography on gelatin-Sepharose 4B as described previously (8). Only one precipitate was demonstrated, when investigated by crossed immunoelectrophoresis in rabbit antibodies against human serum proteins (DAKO, Copenhagen, Denmark). Ten milliliters of gelatin-Sepharose 4B was used to purify 0.5 g of fibrinogen.

Antifibrinogen antibodies. Rabbit antibodies against human fibrinogen were obtained from DAKO.

Fibrinogen-linked Sepharose 4B. Fibrinogen-linked Sepharose 4B was prepared by linking 0.9 g of human fibrinogen to 30 g of CNBr-activated Sepharose 4B by the procedure given by the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden).

Clumping assay. Clumping of staphylococci in the presence of purified fibrinogen was performed by the slide test as described previously (13). Briefly, staphylococci were scraped off truche agar and emulsified in a drop of saline on a clean microscope slide until the consistency was like that of thick cream. Five microliters of human fibrinogen serially diluted in saline or saline as a control was added and immediately mixed. A reaction was positive when visible clumping occurred within 30 s.

Inhibition of fibrinogen-induced *S. aureus* clumping. For the selection of substances useful for desorption components from *S. aureus* bound to fibrinogen-Sepharose 4B, different substances were assessed for their ability to inhibit the clumping of strains E1369 and E2371 in the presence of fibrinogen (3 g/liter). The staphylococci were emulsified in the substance to be tested, and the clumping reaction was performed as described above. The following substances were tested: NaCl, $CaCl_2$, KSCN, carbamide, D-glucose, sucrose, and L-lysine (Table 1).

Affinity chromatography on fibrinogen-linked Sepharose 4B. Fibrinogen-linked Sepharose 4B in a column (3.1 cm^2 by 33 cm) was equilibrated with Tris-hydrochloride (0.05 M)–NaCl (0.5 M; pH 7.7). The samples applied to the column were dissolved in equilibration buffer in the absence or presence of basic pancreatic trypsin inhibitor (Trasylol; Bayer, Leverkusen, Federal Republic of Germany) at a final concentration of 20 μM. All fractions were quantitated by absorbance at 280 nm and by fused rocket immunoelectrophoresis in polyspecific staphylococcal antibodies (see below).

Gel filtration on Sepharose CL6B. The M_r of components eluted from the fibrinogen-Sepharose CL6B was determined by gel filtration. Sepharose CL6B (Pharmacia Fine Chemicals) in a column (6.8 cm^2 by 100 cm) was equilibrated with Tris-hydrochloride (0.05 M)–NaCl (0.1 M; pH 7.7). The sample was applied to the column in a 0.5-volume equilibration buffer. All fractions eluted were investigated by fused rocket immunoelectrophoresis. High-molecular-weight markers (Pharmacia Fine Chemicals) were applied as described above for the calibration of the column, and the fractions were quantitated by absorbance. The markers used were blue dextran 2000, M_r 2×10^6; thyreoglobulin, M_r 6.69

TABLE 1. Inhibition of *S. aureus* clumping[a]

Substance	Concn (M)	Inhibition of the following strains[b]:	
		E1369	E2371
NaCl	2	+	−
	1	+	−
	0.5	+	+
$MgCl_2$	2	−	−
	1	−	−
	0.5	−	−
	0.25	−	−
	0.10	−	−
	0.05	+	−
	0.01	+	+
$CaCl_2$	2	−	−
	1	+	+
KSCN	2	−	−
	1	−	−
	0.5	+	−
	0.25	+	+
Carbamide	8	−	−
	4	+	−
	2	‡	‡
D-Glucose	2	‡	‡
Sucrose	2	+	−
	1	+	+
L-Lysine	2	−	−
	1	+	−
	0.5	+	+

[a] The slide test was performed in the presence of different substances diluted in 0.154 mM NaCl. The fibrinogen concentration used was 3 g/liter.
[b] +, Positive slide test; −, negative slide test.

$\times 10^5$; ferritin, M_r 4.4×10^5; catalase, M_r 2.1×10^5; aldolase, M_r 1.56×10^5.

Immunoelectrophoretic methods. Fractions from the chromatographic experiments were investigated by fused rocket immunoelectrophoresis (45). Fifteen microliters of each fraction was applied in wells, and the fractions were allowed to diffuse for 30 min. Electrophoresis was performed for 18 h at 2 V/cm into polyspecific antibodies raised in rabbits (100 to 600 $\mu g/cm^2$ of gel). Crossed immunoelectrophoresis was performed as described previously (14, 16). Briefly, 1 to 15 μl of antigen solution was applied to the well, and first-dimension electrophoresis was performed for 70 min at 10 V/cm. Second-dimension electrophoresis was performed for 18 h at 2 V/cm into polyspecific antibodies raised in rabbits (100 to 600 $\mu g/cm^2$ of gel). Crossed-line immunoelectrophoresis and tandem crossed immunoelectrophoresis was carried out as described by Krøll (33, 34). Crossed affinoimmunoelectrophoresis (3) was performed by the inclusion of lectins coupled to Sepharose gels or Sepharose 4B (control) in either the first-dimension gel (100 $\mu g/cm^2$ of gel) or in the intermediate gel (100 $\mu g/cm^2$ of gel). *Helix pomatia* (Roman snail)-lectin-Sepharose 6MB, wheat-germ-lectin-Sepharose 6MB, concanavalin A-Sepharose, and Sepharose 4B (control) were obtained from Pharmacia Fine Chemicals. Agarose (1%; Litex, Copenhagen, Denmark) and Tris-barbital buffer (pH 8.6; ionic strength, 0.02) were used. The immunoprecipitates were stained with Coomassie brilliant blue.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the use of PAA 4/30 gradient gels (Pharmacia Fine Chemicals), and the electrophoresis was carried out by the procedure given by the manufacturer. Coomassie brilliant blue staining was performed as described by Weber and Osborn (49). M_r was determined by the use of reference proteins (high-molecular-weight kits; Pharmacia Fine Chemicals).

Western blot analysis. Western blot analysis was carried out as described previously (5). Electrophoretic transfer of protein from unstained SDS-PAGE gels was performed by a modification of the technique described by Towbin et al. (48). A nitrocellulose-gel sandwich was assembled, and the transfer was performed to the nitrocellulose paper (HAWP 2930; pore size, 0.45 μm; Millipore) at 20°C for 18 h at 24 V in Tris-hydrochloride (25 mM)–glycine (0.192 M; pH 8.4) containing methanol (4.9 M). After transfer, the remaining binding sites on the paper were blocked by incubation with Tween 20 (2% [wt/vol]) for 2 min at 20°C. The nitrocellulose sheets were then incubated for 1 h at 20°C with rabbit antibodies against staphylococcal antigens diluted 1/600 in Na_2HPO_4 (8 mM)-KCl (0.2 mM) containing Triton X-100 (1% [wt/vol]-bovine albumin (0.145 mM) (buffer A). The nitrocellulose sheet was then washed for 10 min in five changes of buffer A without albumin, and the sheet was incubated for 1 h at 20°C with peroxidase-conjugated goat anti-rabbit IgG antibody (TAGO, Burlingame, Calif.) diluted 1/1,000 to 1/2,000 in buffer A. The washing was repeated as described above, and the sheet was incubated in CH_3COONa (10 mM) (pH 5.2) for 2 min at 20°C. Finally, the peroxidase activity was detected by incubation for 10 min at 20°C in the dark with CH_3COONa (10 mM; pH 5.2) containing H_2O_2 (5 mM), *N*,*N*-dimethylformamide (0.76 M), and 3-amino-9-ethylcarbazole (3 mM). The enzyme reaction was stopped by washing the sheet in distilled water.

Determination of amino acids, amino sugars, and carbohydrate. Samples for amino acid determination were hydrolyzed in duplicate in 6 M HCl containing 0.1% phenol at 110°C for 24, 48, and 72 h in sealed, evacuated ampoules flushed with nitrogen. Values for serine and threonine were corrected for loss during hydrolysis; values for valine, isoleucine, and phenylalanine were taken after 72 h of hydrolysis. The content of cysteine and cystine was determined after oxidation with performic acid (38). Samples for the determination of amino sugars were hydrolyzed for 6 h at 110°C in 4 M HCl. Analyses were performed with a Waters high-pressure liquid chromatographic amino acid analysis system by ion-exchange chromatography and postcolumn derivatization with *o*-phthalaldehyde. With the nonhalide linear pH gradient proposed by the manufacturer, galactosamine is separated from the amino acids whereas glucosamine coelutes with phenylalanine. A steeper gradient separated these residues and was used for the quantitation of glucosamine. No amino sugars other than glucosamine and galactosamine were included in the standard.

Carbohydrate was determined relative to mannose by the method of Dubois et al. (9) in reduced scale (1 to 2 μg). Four samples of the preparation were analyzed with a resulting standard error of the mean of 10%.

Competition of fibrinogen-induced *S. aureus* clumping. Components eluted from the fibrinogen-Sepharose 4B column were tested for their ability to inhibit the fibrinogen-induced clumping in the slide test. Purified fibrinogen was serially diluted in saline in the absence or presence of the eluted component and incubated for 15 min at room temperature before the clumping tests were performed.

RESULTS

Inhibition of the fibrinogen-induced *S. aureus* clumping. The ability of different substances to inhibit clumping of *S. aureus* E1369 and E2371 in the presence of fibrinogen is

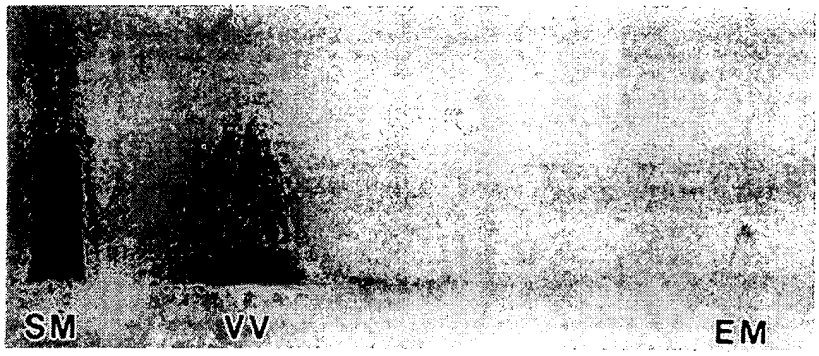


FIG. 1. Elution profile of a water-soluble supernatant after sonication of *S. aureus* E1369 fractionated on fibrinogen-Sepharose 4B. Fused rocket immunoelectrophoresis (anode at the top) of 15 μl of each fraction in the wells was run into polyspecific antibody against sonicated strain E1369 (300 μg/cm² of gel). An immunoprecipitate can be seen after elution with 1 M $MgCl_2$ was started. Abbreviations: SM, starting material; VV, void volume; EM, eluted material.

shown in Table 1. $MgCl_2$ was the most potent inhibitor of the clumping reaction, and 1 M $MgCl_2$ was chosen for desorption of bound components from fibrinogen-Sepharose 4B.

Affinity chromatography on fibrinogen-linked Sepharose 4B. The first experiment was carried out by application of 0.4 g of protein from the water-soluble phase of sonicated whole cells of *S. aureus* E1369 to the column. Most of the applied protein appeared in the void volume of fibrinogen-Sepharose 4B as estimated by the absorbance at 280 nm and fused rocket immunoelectrophoresis (Fig. 1). After elution with 1 M $MgCl_2$, only one precipitate was identified by fused rocket immunoelectrophoresis in polyspecific rabbit antibodies against sonicated preparations from the same strain (Fig. 1). The absorbance of the peak at 280 nm did not differ from that of the base line. The eluted material was pooled, dialyzed twice against distilled water for 24 h each, and lyophilized. The yield after lyophilization was 0.075% (0.3 mg).

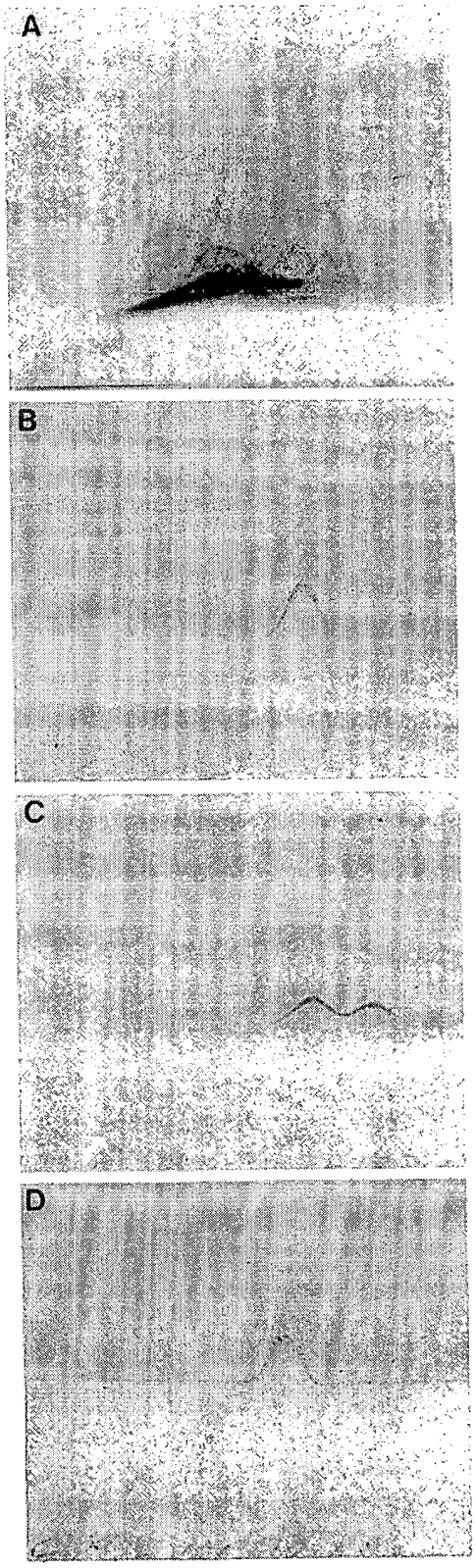
In this affinity chromatography there was no basic pancreatic trypsin inhibitor either in the sample or in the equilibration buffer. In the second experiment with the same column a yield of below 5% that of the first was obtained. It was suggested that proteolytic activity in the sample degraded the fibrinogen linked to Sepharose 4B. After preparation of a new fibrinogen-Sepharose 4B, basic pancreatic trypsin inhibitor (20 μM) was added to the sample and the equilibration buffer. The new column was used in 18 experiments, and the capacity was only decreased to about 85 to 90%.

Table 2 summarizes the experiments performed by application of the different bacterial extracts on the fibrinogen-Sepharose 4B column. The eluted fractions were investigated by fused rocket immunoelectrophoresis. The volume corresponding to the immunoprecipitates in positive fused rocket immunoelectrophoretic assays was concentrated 100 times by lyophilization and investigated by crossed immunoelectrophoresis. In all experiments in which sonicated preparations of cells or cell walls of *S. aureus* were applied, a visible immunoprecipitate was obtained by both fused rocket immunoelectrophoresis and crossed immunoelectro-

TABLE 2. Affinity chromatography on fibrinogen-Sepharose 4B of different water-soluble staphylococcal preparations[a]

Strain	Preparation	Amt of protein applied (mg)	Precipitate in eluted fractions[b]	Visible precipitates after lyophilization[c]	Amt eluted (mg)
S. aureus					
E1369	Sonicated whole cells	400	+	One	0.2
E2371	Sonicated whole cells (1)	400	+	One	1.6
E2371	Sonicated whole cells (2)	400	+	One	1.4
E2476	Sonicated whole cells	400	+	One	0.3
E1346	Sonicated whole cells	400	+	One	0.1
E1430	Sonicated whole cells	400	+	One	<0.1
E1369	Sonicated cell walls	84	+	One	<0.1
E2371	Sonicated cell walls	52	+	One	<0.1
E1369	Sonicated L-forms	12	ND[d]	None	ND
E1369	Extracellular products	174	None	One	ND
E2371	Extracellular products	102	None	One	ND
S. epidermidis					
A1271/76	Equal volumes of sonicated whole cells	400	None	None	ND
A1388/76	Equal volumes of sonicated whole cells	400	None	None	ND
A1394/76	Equal volumes of sonicated whole cells	400	None	None	ND
A1389/76	Equal volumes of sonicated whole cells	400	None	None	ND

[a] Elution was performed with 1 M $MgCl_2$.
[b] Fused rocket immunoelectrophoresis.
[c] Crossed immunoelectrophoresis after dialysis and lyophilization.
[d] ND, Not determined.



phoresis (Table 2). No eluted material was obtained when truche medium alone was applied to the column. The highest yield of eluted material was obtained by the use of sonicated material from strain E2371 (Table 2). Application of extracellular products from strains E1369 and E2371 also resulted in a visible precipitate, but the yield was so small that it was only detectable after it was concentrated (Table 2). No eluted material could be detected when extracts of L-forms from strain E1369 or *S. epidermidis* were applied to the column (Table 2).

Characterization of the eluted component by crossed immunoelectrophoresis. More than 50 immunoprecipitates could be identified by crossed immunoelectrophoresis, when a sonicate of strain E2371 was used as sample, and polyspecific antibodies against the four phage groups of *S. aureus* were used as antibodies (Fig. 2A). When the eluted components released in the different fibrinogen-Sepharose 4B experiments were investigated by crossed immunoelectrophoresis in polyspecific *S. aureus* antibodies, they all revealed only one precipitate in the α-globulin region of serum proteins (Fig. 2B). This precipitate was identified in all *S. aureus* antibodies (i.e., antibodies raised against sonicated preparations of whole cells; cell walls; extracellular products; and whole, heat-killed cells), but the precipitate was not seen when antibody against sonicated *S. epidermidis* was used (data not shown). The precipitates obtained from experiments with different strains of *S. aureus*, from cell walls, as well as from extracellular products were shown to be immunochemically identical. The immunological identity was evaluated by tandem crossed immunoelectrophoresis and crossed-line immunoelectrophoresis (Fig. 2C and D). The eluted component did not contain antigenic sites from fibrinogen, as no precipitates were identified by crossed immunoelectrophoresis in antifibrinogen antibodies. The eluted *S. aureus* antigen bound to all the three lectins investigated, both with the lectins included in the first-dimension gel as well as in the intermediate gel of a crossed immunoelectrophoresis (data not shown).

Gel filtration of the eluted component. The sample applied was 0.2 mg of eluted component from an affinity chromatographic experiment in which sonicated whole cells of strain E2371 were used. This sample was applied to the Sepharose CL6B column, and the eluted fractions were investigated by fused rocket immunoelectrophoresis with rabbit antibodies against *S. aureus* cell walls. Fused rocket immunoelectrophoresis showed only one immunoprecipitate, and M_r was estimated to be 420,000 (data not shown).

FIG. 2. (A) Crossed immunoelectrophoresis of sonicated *S. aureus* E2371 antigen into polyspecific antibody against sonicated *S. aureus* E2371. In the first dimension (anode at the right), 40 μg of sonicated antigen from strain E2371 was applied to the well. The second dimension (anode at the top) contained polyspecific antibody against sonicated strain E2371 (200 μg/cm² of gel). The intermediate gel contained saline. Fifty-seven immunoprecipitates can be identified. (B) In the first dimension, 0.5 μg of component (strain E2371) eluted from the fibrinogen column was applied to the well. The second dimension and the intermediate gel are as described in (A). (C) Tandem crossed immunoelectrophoresis. Components (0.5 μg; strains E2371 and E1369, respectively) eluted from the fibrinogen column were applied to the wells. The second dimension and intermediate gels are as described in (A). (D) Crossed line immunoelectrophoresis. The immunoplate was identical to that described in (A), but instead of saline, the intermediate gel contained the component (strain E1369) eluted from fibrinogen-Sepharose 4B (25 μg/cm² of gel).

SDS-PAGE and Western blot analysis of the eluted component. SDS-PAGE with and without reduction of the eluted component from strain E2371 revealed only one protein band with an M_r of 360,000 (Fig. 3). Western blot analysis with polyspecific *S. aureus* antibodies revealed the same band (Fig. 4), but not when the polyspecific *S. epidermidis* antibody was used.

Binding of the eluted component to fibrinogen demonstrated by crossed immunoelectrophoresis. After incubation of the eluted component with fibrinogen, crossed immunoelectrophoresis in antibody against *S. aureus* cell walls revealed a changed morphology of the immunoprecipitate with an increasing cathodic tail and a decreased electrophoretic mobility of the component as the concentration of fibrinogen increased (Fig. 5).

Chemical analysis of the eluted component from strain E2371. The amino acids, amino sugars, and carbohydrates were determined from a preparation of the eluted component obtained from sonicated preparation of whole cells of strain E2371 (1) (Table 2). The eluted component was found to have a carbohydrate content of about 20% weight (Table 3). Aspartic acid, serine, and glycine were the predominant amino acids, whereas no cysteic acid was identified after oxidation (Table 3). A high amount of glucosamine and much less galactosamine were found.

Competition of the fibrinogen-induced clumping of *S. aureus* by the eluted components. Clumping of strains E1369 and E2371 in the presence of fibrinogen was significantly reduced when the eluted component from strain E2371 was present (Table 4). The minimal concentration of fibrinogen necessary to induce clumping was increased from 0.014 to 0.23 μM and from 0.057 to 1.8 μM from strains E1369 and E2371, respectively (Table 4).

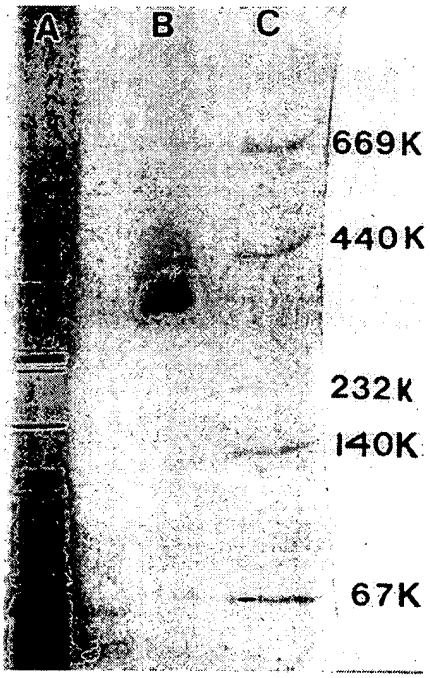


FIG. 3. SDS-PAGE of the component (strain E2371) eluted from fibrinogen-Sepharose 4B and sonicated *S. aureus* E2371. (A) Sonicated *S. aureus* antigen (64 μg; strain E2371) was applied (anode at the bottom). (B) Eluted component (3 μg) was applied. One main band can be seen. (C) Molecular mass standards. K, Kilodaltons.

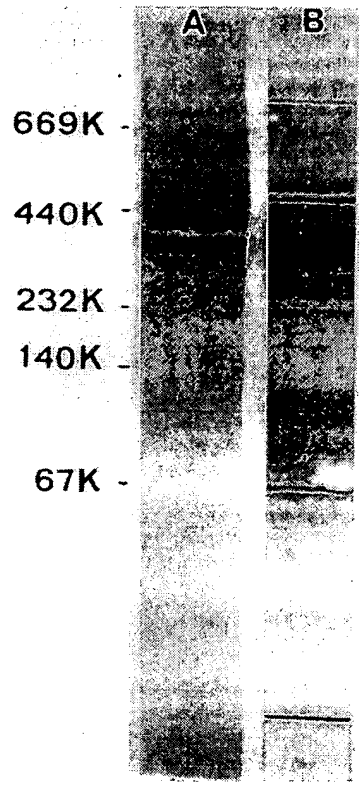


FIG. 4. Western blot analysis after SDS-PAGE of the component (strain E2371) eluted from fibrinogen-Sepharose 4B and cell walls from *S. aureus* E2371. (A) Eluted component (2 μg) was applied (anode at the bottom). One band (M_r of about 360,000) can be seen. (B) Cell wall (52 μg) from strain E2371 was applied. The antibodies used were polyspecific antibodies against *S. aureus* cell walls. K, Kilodaltons.

DISCUSSION

In the present study, it was possible to purify immunochemically identical components from water-soluble extracts of five different *S. aureus* strains by means of affinity chromatography on fibrinogen-linked Sepharose 4B. The affinity of these components for human fibrinogen was high, since the binding took place in the presence of 0.5 M NaCl. The purity of the component was demonstrated by crossed immunoelectrophoresis and Western blot analysis (Fig. 1 and 3). Only one precipitate and one band could be identified by use of different polyspecific *S. aureus* antibody preparations. The component could not be isolated from similar extracts of *S. epidermidis*.

The isolated component seems to be a cell wall component (Table 2). It could be isolated from both sonicated whole cells and sonicated cell walls but not from sonicated preparations of wall-defective *S. aureus* (Table 3). Antibodies against the component were obtained from rabbit antisera prepared by hyperimmunization intracutaneously with sonicated preparations and purified cell walls from *S. aureus* (Fig. 2 and 4). Furthermore, antibody prepared by a short immunization intravenously with whole, heat-killed bacteria

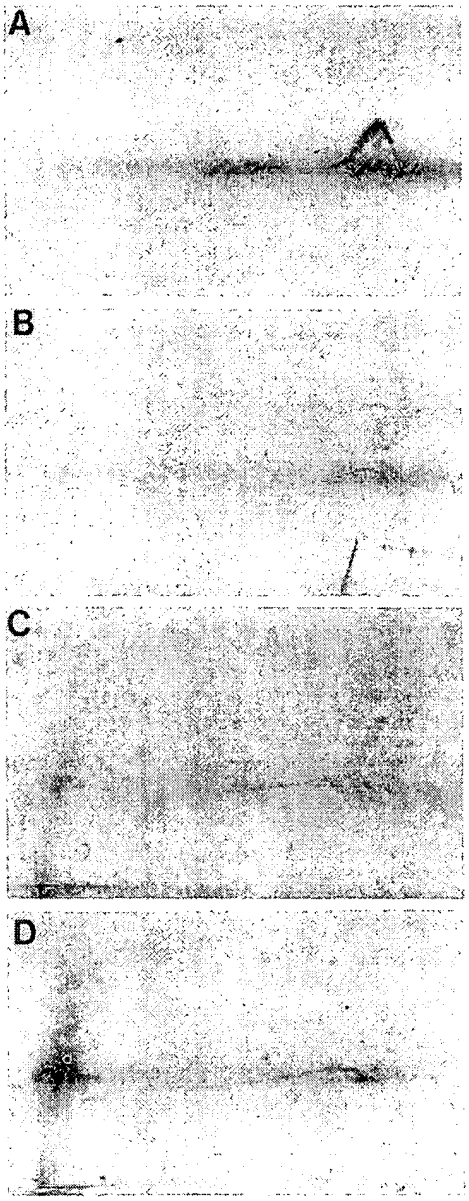


FIG. 5. Demonstration of a complex between the component eluted from the fibrinogen column and fibrinogen by crossed immunoelectrophoresis. (A) In the first dimension (anode to the right), 0.6 μg of component (strain E2371) eluted from fibrinogen-Sepharose 4B was applied to the well. The second dimension (anode at the top) contained polyspecific antibodies against *S. aureus* cell walls (100 μg/cm² of gel). The intermediate gel contained saline. (B) through (D) In the first dimension, 0.6 μg of component (strain E2371) eluted from the fibrinogen column mixed with 2.5, 12.5, and 62.5 μg of human fibrinogen, respectively (incubated at 37°C for 20 min), was applied to the wells. The second dimension and the intermediate gel were as described in (A). After incubation with increasing amounts of fibrinogen, the immunoprecipitate decreased electrophoretic mobility and changed morphology with an increasing cathodic tail.

precipitated the component. Small amounts of the component were isolated from extracellular products of *S. aureus* (Table 2), and polyspecific antibodies against extracellular products precipitated this component. These findings might be explained by the secretion of the component into the environment during growth of the bacteria, but they might also be explained by the liberation of the component to the environment from autolyzed bacteria.

The isolated component seems to be a hitherto uncharacterized cell wall component of *S. aureus*. Protein A has an M_r of about 42,000 (2), and both peptidoglycan and teichoic acid mainly are composed of polysaccharide (42, 47). The relative molecular mass was about 360,000 and 420,000, as determined by SDS-PAGE and gel filtration, respectively. In both methods globular proteins with no carbohydrate were used as references. The chemical composition (Table 3) shows a relatively high content of carbohydrate (20% of the total weight). The findings by SDS-PAGE of only one band under reduced conditions, in agreement with the lack of cystine (Table 3), points toward a primary structure of one polypeptide chain. However, it seems unlikely that a bacterium produces a polypeptide chain of that magnitude. Based on the chemical composition, the isolated component may contain peptidoglycan fragments. Therefore, it seems possible that the component is a complex containing one or several fibrinogen-binding proteins linked to peptidoglycan fragments by binding not broken by SDS. Such a structure also could probably explain the difference in M_r determined by the two methods, in which globular proteins were used as references. Wheat-germ lectin and *H. pomatia* lectin have a high specificity for *N*-acetylglucosamine and *N*-acetylgalactosamine, respectively, whereas concanavalin A is a relatively nonspecific carbohydrate binder. The affinity of the purified glycoprotein for all three lectins indicates that the molecule contains both *N*-acetylglucosamine and *N*-acetylgalactosamine residues.

TABLE 3. Contents per milligram of the eluted component (*S. aureus* E2371)[a]

Amino acid	Preparation (nmol)
Aspartic acid	1,239
Threonine	297
Serine	843
Glutamic acid	463
Proline	234
Glycine	546
Alanine	360
Cysteic acid[b]	0
Valine	333
Methionine	40
Isoleucine	227
Leucine	237
Tyrosin	135
Phenylalanine	105
Histidine	59
Lysine	316
Arginine	74
Tryptophan	ND[c]
Glucosamine	937
Galactosamine	120

[a] The weight of the identified amino acids and hexasamines was 766 μg. The weight of carbohydrates ± standard error of the mean was 190 ± 20 μg.
[b] After oxidation.
[c] ND, Not determined.

TABLE 4. Competition of *S. aureus* clumping[a]

Strain	Dilution	Results of slide test at the following reciprocals of fibrinogen dilutions[b]:											
		1	2	4	8	16	32	64	128	256	512	1024	2048
E1369	A	+	+	+	+	+	+	+	+	+	+	−	−
	B	+	+	+	+	+	+	−	−	−	−	−	−
E2371	A	+	+	+	+	+	+	+	+	−	−	−	−
	B	+	+	+	−	−	−	−	−	−	−	−	−

[a] The slide test was performed with fibrinogen (2.5 g/liter) twofold diluted in 0.154 mM NaCl (A) or in 0.154 mM NaCl (B) containing 0.05 g of the fibrinogen-binding component isolated from a sonicated preparation of strain E2371 per liter.
[b] +, Positive slide test; −, negative slide test.

The clumping factor from *S. aureus* has been suggested to be a protein or a peptide complex, as it is destroyed by proteolytic enzymes (46). It has been shown to be a surface component and not to be present in the staphylococcal L-forms (12), as was found for the purified component in the present study (Table 2). The component could not be isolated from *S. epidermidis*, which has no clumping capacity (12), and does not bind to fibrinogen (36). A high affinity between fibrinogen and *S. aureus* has been proposed from the finding that clumping of *S. aureus* occurs with only 20 molecules of fibrinogen per one staphylococcus (23), giving a dissociation constant (K_d) of about 10^{-8}, as found by the use of ^{125}I-labeled human fibrinogen and a steady-state binding system (24). The purified component binds strongly to fibrinogen, but this does not prove that it is identical to the clumping factor. However, the fact that binding of the purified component to fibrinogen before its addition to *S. aureus* results in inhibition of the clumping (Table 4) strongly suggests that the isolated component competes with the clumping factor on whole *S. aureus* for the binding site on fibrinogen and thus is identical to the clumping factor or contains this factor.

Results of studies on the relation of clumping factor and staphylococcal virulence are often contradictory, but most studies are based on comparisons between *S. aureus* strains with or without clumping factor activity (1, 30, 32, 46). The isolation of clumping factor by this one-step method makes it possible to investigate the biological and immunological activities of the clumping factor.

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Is the GehD Lipase from *Staphylococcus epidermidis* a Collagen Binding Adhesin?*

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The opportunistic human pathogen *Staphylococcus epidermidis* is the major cause of nosocomial biomaterial infections. *S. epidermidis* has the ability to attach to indwelling materials coated with extracellular matrix proteins such as fibrinogen, fibronectin, vitronectin, and collagen. To identify the proteins necessary for *S. epidermidis* attachment to collagen, we screened an expression library using digoxigenin-labeled collagen as well as two monoclonal antibodies generated against the *Staphylococcus aureus* collagen-adhesin, Cna, as probes. These monoclonal antibodies recognize collagen binding epitopes on the surface of *S. aureus* and *S. epidermidis* cells. Using this approach, we identified GehD, the extracellular lipase originally found in *S. epidermidis* 9, as a collagen-binding protein. Despite the monoclonal antibody cross-reactivity, the GehD amino acid sequence and predicted structure are radically different from those of Cna. The mature GehD circular dichroism spectra differs from that of Cna but strongly resembles that of a mammalian cell-surface collagen binding receptor, known as the α_1 integrin I domain, suggesting that they have similar secondary structures. The GehD protein is translated as a preproenzyme, secreted, and post-translationally processed into mature lipase. GehD does not have the conserved LPXTG C-terminal motif present in cell wall-anchored proteins, but it can be detected in lysostaphin cell wall extracts. A recombinant version of mature GehD binds to collagens type I, II, and IV adsorbed onto microtiter plates in a dose-dependent saturable manner. Recombinant, mature GehD protein and anti-GehD antibodies can inhibit the attachment of *S. epidermidis* to immobilized collagen. These results provide evidence that GehD may be a bi-functional molecule, acting not only as a lipase but also as a cell surface-associated collagen adhesin.

Staphylococcus epidermidis is now recognized as an important nosocomial pathogen. In the past 20 years it has emerged as a frequent cause of infections associated with indwelling devices such as catheters, artificial heart valves, and orthopedic implants (1). In certain populations such as low birth weight infants and immuno-compromised patients *S. epidermidis* can be a prominent source of morbidity and mortality (2).

The molecular mechanisms of pathogenesis of *S. epidermidis* disease are not well understood, but as with most infections, bacterial adherence to host surfaces is recognized as the first crucial step in the infection process and a prerequisite for colonization. A two-step process of *S. epidermidis* adherence is often described in which the first step is bacterial attachment to the biomaterial, and the second step includes microbial proliferation, intercellular adhesion, and biofilm formation. Almost all *S. epidermidis* strains are able to attach to native abiotic surfaces (3–6). However, any foreign material implanted into the human body is quickly coated with various plasma proteins such as fibrinogen, fibronectin, and vitronectin (7, 8), and *Staphylococcus aureus*, which is also a common cause of biomaterial centered infections, appears to adhere to this protein coat via adhesins of the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)[1] type.

Analysis of the adherence behavior of *S. epidermidis* suggests that this organism also expresses MSCRAMMs. In fact, a gene encoding a fibrinogen binding MSCRAMM (*sdrG*, also called *fbe*) was cloned and sequenced from *S. epidermidis* (9). SdrG, a 119-kDa MSCRAMM, has a structural organization similar to the clumping factor (ClfA) from *S. aureus* and specifically recognizes the N-terminal region of the fibrinogen Bβ chain (10). In addition, the autolysin AtlE, necessary for *S. epidermidis* attachment to polystyrene, was shown to specifically bind to biotin-labeled vitronectin (11). These data indicate that *S. epidermidis*, similarly to *S. aureus*, may express specific MSCRAMMs that mediate cell attachment to host protein-conditioned surfaces.

In the present communication, we report that the GehD (12) lipase binds to collagen type I, II, and IV and may mediate the adherence of *S. epidermidis* cells to immobilized collagens. We identified GehD probing a *S. epidermidis* expression library with labeled collagen type I and monoclonal antibodies generated against the *S. aureus* collagen-binding protein, Cna. Staphylococcal lipases have been implicated as possible virulence factors in localized infections such as absesses (13–15), and there is evidence that they are highly expressed during infection in a murine model (16). The contribution of these

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[1] The abbreviations used are: MSCRAMM, microbial surface components recognizing adhesive matrix molecules; PBS, phosphate-buffered saline; mAb, monoclonal antibody (Ab); BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay.

APPENDIX B

enzymes to virulence is not clearly understood, although lipases may be important for the colonization and persistence of organisms on the skin (17).

Another class of proteins that function as collagen binding adhesion receptors are the mammalian integrins. These proteins mediate the attachment of eukaryotic cells to the extracellular matrix. The integrins are transmembrane $\alpha\beta$ heterodimeric proteins that mediate cell-cell and cell-matrix interactions of mammalian cells. In this extensive family of proteins, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the primary collagen binding integrins. Within the α subunit of the collagen binding integrins, the ligand binding region is called I domain (33). Our data predict that mature GehD may adopt a structure that resembles that of the integrin α_1 I-domain.

The data described here show that the GehD lipase binds to collagens and may promote *S. epidermidis* attachment to immobilized collagens. Our data indicate that the GehD lipase may be a bifunctional molecule, acting as a glycerol ester hydrolase and a collagen adhesin.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—*S. epidermidis* strains 146, 9491, 12228, 14852, and 14990 were obtained from the ATCC collection. *S. epidermidis* 9, 2J24 (*gehC*::*ermC*), and KIC82 (*gehD*::*ermC*) were created by Christopher M. Longshaw (12). *S. aureus* Cowan 1 spa::*tet*^{R} strain was generously donated by T. Foster (University of Dublin, Ireland). All strains were grown in brain heart infusion or tryptic soy broth media (Difco) at 37 °C overnight. For the monoclonal antibody reactivity assays, bacteria were harvested and re-suspended in phosphate-buffered saline (PBS), pH 7.4 (140 mM NaCl, 270 μM KCl, 430 μM Na_2HPO_4, 147 μM KH_2PO_4) 0.02% sodium azide, washed, and adjusted to a cell density of 10^{10} cells/ml using a standard curve relating the A_{600} to the cell number determined by counting cells in a Petroff-Hausser chamber. The cells were then heat-killed at 88 °C for 10 min.

For all other assays, overnight cultures were diluted 1:1000 into fresh tryptic soy broth media, and the resultant culture was incubated until it reached logarithmic growth phase (A_{600} 0.3–0.6). Bacteria were then harvested by centrifugation and used in attachment or Western assays.

Library Construction—A *S. epidermidis* 9491 λZAP Express (Stratagene) expression library was constructed as follows. *S. epidermidis* 9491 chromosomal DNA was partially digested with *Mbo*I, and the fragments corresponding to 3–11 kilobases were isolated and purified. The purified fragments were ligated to the ZAP Express® (Stratagene) vector, predigested with *Bam*HI, and dephosphorylated with CIAP (calf intestinal alkaline phosphatase). The resultant ligation product was packaged into phage particles using the Gigapack III Gold (Stratagene)-packaging extract. The obtained library was amplified and screened using the *Escherichia coli* XL1-Blue MRF′ strain. Clones of interest were excised from the λ ZAP Express® phage using the ExAssist® helper phage to generate the pBK-CMV phagemid vector packaged as filamentous phage particles. The filamentous phage stock was used to infect the *E. coli* XLOLR strain. The resultant colonies carrying the excised pBK-CMV phagemid vector were used for subsequent subcloning and dideoxy sequencing of the cloned inserts.

A DNA fragment encoding the mature domain of the GehD lipase was PCR-amplified from *S. epidermidis* 9491 genomic DNA. The oligonucleotides primers 5′-TTT GAA TTC TGC GCA AGC TCA ATA TAA and 5′-TTT GCG GCC GCT ATC GCT ACT TAC GTG TAA were used to amplify the fragment designated as mature GehD. Constructs generated by PCR were cloned into the pETBlue-2 System using the *E. coli* NovaBlue strain as a cloning host and the *E. coli* Tuner (DE3) pLacI strain as the expression host.

Large scale expression and preparation of recombinant proteins were as described previously using HiTrap nickel-chelating chromatography (10). Protein concentrations were determined from the absorbance at 280 nm as measured on a Beckman Du-70 UV-visible spectrophotometer. The molar extinction coefficient of the proteins was calculated using the method of Pace *et al.* (18).

Labeling of Proteins—Purified collagen I (Vitrogen®, Cohesion, Palo Alto CA) was labeled with digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (digoxigenin) (Roche Molecular Biochemicals) according to the manufacturer's instructions.

To label recombinant proteins with biotin, 7.5 mg of sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin; Pierce) was dissolved in 100 μl of dimethyl sulfoxide (Me_2SO) and combined with 0.5 mg of recombinant protein in PBS. The total reaction (1 ml volume) was incubated in an end-over-end rotator at room temperature for 2 h then dialyzed against PBS and stored at 4 °C.

Library Screens—Digoxigenin-labeled collagen or mAbs 11H11 and 1F6 (19) were used to screen the *S. epidermidis* 9491 λZAP Express (Stratagene) expression library. The library was plated using standard methods according to the vector manufacturer's instructions (Stratagene). After blocking additional protein binding sites on the filter lifts with a solution containing 3% (w/v) bovine serum albumin (BSA) in TBST (0.15 M NaCl, 20 mM Tris-HCl, 0.05% (v/v) Tween 20, pH 7.4), digoxigenin-labeled collagen (0.5 μg/ml in TBST) was incubated with the filters. The bound digoxigenin-labeled collagen was incubated with anti-digoxigenin Fab conjugated to alkaline phosphatase (1:5000 in TBST, Roche Molecular Biochemicals). When mAbs 11H11 or 1F6 (1:500 in TBST) were used as probes, goat anti-mouse antibodies (1:4000 in TBST) conjugated to alkaline phosphatase (Bio-Rad) were used as secondary antibodies. Clones expressing collagen-binding proteins were identified by developing the membranes with 5-bromo-4-chlor-3-indoyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride (Bio-Rad).

Enzyme-linked Immunosorbent Assay (ELISA)—To test the reactivity of the mAbs generated against bacterial surface proteins, microtiter wells (Dasit, Milan, Italy) were coated overnight at 4 °C with 2 μg of human fibronectin in 100 μl of 50 mM sodium carbonate, pH 9.5, to provide a surface for bacterial attachment. The wells were washed five times with 10 mM sodium phosphate buffer, pH 7.4, containing 0.13 M NaCl and 0.1% (v/v) Tween 20 (PBST), and additional protein binding sites were blocked with a solution of 2% (w/v) BSA in PBS. Suspensions of 1×10^8 cells of *S. epidermidis* or *S. aureus* Cowan 1 *spa*::*tet*^{R}) whole cells were added and incubated for 2 h at room temperature followed by 5 washes with PBS to remove unbound cells. Solutions of 2 μg of each monoclonal antibody in 100 μl of 2% (w/v) BSA in PBS were added, incubated for 2 h at room temperature, washed extensively with PBST, and detected with a 1:500 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Dako, Gostrup, Denmark). The conjugated enzyme was incubated with *o*-phenylenediamine dihydrochloride (Sigma) as a substrate, and the color development absorbance was monitored at 492 nm using a microplate reader (Bio-Rad).

To test protein-protein interactions, microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 1 μg of type I collagen in 100 μl of PBS/well overnight at 4 °C. Wells were then washed 3 times with PBS and blocked with 1% (w/v) bovine serum albumin in PBS for 1 h before the addition of varying concentrations of the biotinylated recombinant protein. After incubation at room temperature for 2 h with gentle shaking, the wells were extensively washed with PBS containing 0.05% (v/v) Tween 20 (PBST). Streptavidin-alkaline phosphatase conjugate (Roche Molecular Biochemicals) was diluted 10,000-fold with blocking buffer and added to the wells. After incubation at room temperature for 45 min, the wells were washed with PBST. For color development, 100 μl of 1.3 M diethanolamine, pH 9.8, containing 1 mg/ml *p*-nitrophenyl phosphate (Sigma) was added to the wells. Absorbance at 405 nm ($A_{405\ nm}$) was measured using a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA) after 1 h of incubation at room temperature. Experiments were performed in triplicate and repeated with independently prepared protein preparations. Binding to BSA-coated wells was considered as background level and subtracted from binding to collagen. Data were presented as the mean value ± S.E. of $A_{405\ nm}$ from a representative experiment ($n = 3$). The effect of antibodies as inhibitors of proteins binding to collagen was examined as described above except that biotinylated proteins were mixed with antibodies at varying ratios and added to the wells.

Circular Dichroism—The secondary structural composition of recombinant proteins was examined by CD spectroscopy. Far UV CD data were collected using a Jasco J720 spectropolarimeter calibrated with a 0.1% (w/v) *d*-10-camphorsulfonic acid solution using a bandwidth of 1 nm and integrated for 4 s at 0.2-nm intervals. All sample concentrations were less than 30 μM in 20 mM Tris-HCl buffer, pH 7.4. Spectra were recorded at ambient temperatures in 0.2-mm path length cuvettes. Thirty scans were averaged for each spectrum, the contribution from the buffer was subtracted, and quantitation of secondary structural elements was performed by deconvolution software provided by University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School (Piscataway, NJ) and D. Greenwood (Softwood Co., Brooksfield, CT). These deconvolution programs (SELCON and VARSLC1) are derived from databases of known protein structures.

Surface Plasmon Resonance Spectroscopy—Analyses were performed

using the BIAcore 1000 system (BIAcore AB, Uppsala, Sweden) as described previously (20). The Cna protein was tested in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4). The α_1 I domain was tested in HBS containing 5 mM β-mercaptoethanol, and mature GehD was tested in both HBS and glycine buffer (50 mM glycine, pH 7.4). Data from the equilibrium portion of the sensorgrams were used for analysis and calculation of the K_D and n.

Preparation of Polyclonal Antibodies—Purified mature GehD was dialyzed in PBS, pH 7.4, before being sent to Rockland Immunochemicals, Inc. (Gilbertsville, PA) for immunization in rabbits and production of polyclonal antisera. IgGs were purified from both immune and preimmune serum by chromatography using protein A-Sepharose (Sigma).

Bacterial Adherence Assays—Microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 1 μg of type I collagen in 100 μl of PBS/well overnight at 4 °C. Wells were then washed 3 times with PBS and then blocked with 1% (w/v) bovine serum albumin in PBS for 1 h before the addition of bacteria. Early log-phase *S. epidermidis* cultures (A_{600} of 0.5) were added, and the plates were incubated for 2 h at room temperature. After gentle washes, adherent cells were fixed with 100 μl of 25% (v/v) aqueous formaldehyde and incubated at room temperature for at least 30 min. The plates were then washed gently, stained with crystal violet, then washed again and read on an ELISA plate reader at 590 nm.

To study inhibition of collagen binding by IgGs, *S. epidermidis* suspensions were preincubated with serial dilutions of purified IgGs in PBS for 2 h at room temperature. The cell suspensions were then transferred to ELISA plates coated with 1 μg of collagen/well, and their ability to attach to collagen was tested as described above.

SDS-PAGE and Western Ligand Blot—For whole-cell SDS-PAGE (21), 2×10^7 *S. epidermidis* (previously treated with lysostaphin) cells or *E. coli* cells were boiled in 2% (w/v) SDS for 3–5 min under reducing conditions and subjected to electrophoresis through a 10% acrylamide gel at 150 V for 45 min. The separated proteins were stained with Coomassie Brilliant Blue.

For Western ligand blot assays, whole cell lysates, or purified proteins were transferred from the polyacrylamide gel onto a nitrocellulose membrane in a semi-dry electroblot system (Bio-Rad). Additional binding sites on the membrane were blocked by incubating in 2% (w/v) BSA in TBST for 2 h at room temperature or overnight at 4 °C followed by three 10-min washes in TBST. The membrane was then incubated at room temperature with 0.5 μg of digoxigenin-labeled collagen/ml TBST for 1 h, washed, and incubated with 1:5000 anti-digoxigenin Fab alkaline-phosphatase conjugate (Roche Molecular Biochemicals) in TBST for 1 h. The membrane was washed, and collagen-binding proteins were visualized with 150 μg of 5-bromo-4-chlor-3-indoyl phosphate *p*-toluidine salt/ml and 300 μg of *p*-nitro blue tetrazolium chloride/ml (Bio-Rad) in carbonate bicarbonate buffer (14 mM Na_2CO_3, 36 mM $NaHCO_3$, 5 mM $MgCl_2 \cdot 6H_2O$, pH 9.8).

RESULTS

Adherence of S. epidermidis 9491 to Extracellular Matrix Proteins—The clinical isolate *S. epidermidis* 9491 was chosen as a prototype strain in our search for new MSCRAMMs. We tested its ability to adhere to immobilized bovine collagen type I, human fibrinogen, and human fibronectin. Each protein was immobilized in microtiter wells, and the bacteria attached to the wells were detected using crystal violet. The results presented in Fig. 1 show that *S. epidermidis* 9491 has the ability to attach to collagen, fibrinogen and fibronectin. Although previous studies have shown that *S. epidermidis* attachment to human fibrinogen is mediated by proteins such as Fbe and SdrG (9, 10), the bacterial components that mediate attachment to collagen or fibronectin were up to this point not identified.

Binding of Monoclonal Antibodies to S. epidermidis Strains—A panel of 22 monoclonal antibodies was previously generated against the *S. aureus* MSCRAMM Cna-(151–318) (19). We explored the possibility that at least some of these mAbs would cross-react with collagen-binding proteins on *S. epidermidis* by examining a panel of strains (*S. epidermidis* 146, 9491, 12228, 14852, and 14990). Two monoclonals, 11H11 and 1F6, cross-reacted with whole cells of all the *S. epidermidis* strains tested. Both of these antibodies were raised against the ligand binding central region of Cna-(151–318). Furthermore,

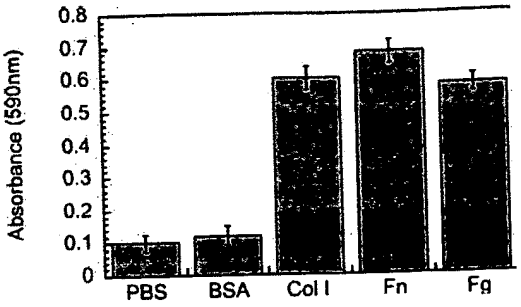

FIG. 1. ***S. epidermidis* can bind to immobilized extracellular matrix proteins.** Log-phase bacterial cultures were washed and incubated in microtiter wells coated with 1 μg of BSA, collagen I (*coll*), fibronectin (*Fn*), and fibrinogen (*Fg*). Attached *S. epidermidis* cells were detected using crystal violet. Values represent the means and S.E. of triplicate wells. This experiment was repeated several times with similar results.

these antibodies were shown to inhibit collagen binding to Cna and recognize conformationally dependent epitopes, presumably located in the ligand binding site of Cna-(151–318) (19). As expected, all of the anti-Cna mAbs bind to *S. aureus* Cowan 1 cells. The *S. epidermidis* strains are recognized only by two antibodies. These results suggest that *S. epidermidis* exposes on its surface proteins that form epitopes similar to those present on Cna and that these proteins are recognized by 1F6 and 11H11.

Construction of an Expression Library and Identification of a New Collagen-binding Protein—We constructed an expression library ligating *Mbo*I partially digested, size-selected genomic DNA from *S. epidermidis* 9491 to *Bam*HI-digested λZAP Express II® vector. Using mAbs 1F6 and 11H11 as well as digoxigenin-labeled collagen, we screened ~690,000 plaques. We isolated three clones that reacted with each mAb and labeled collagen. DNA sequencing of the excised phagemids revealed that 2 of the clones were identical, and the third had an additional 36 bp of upstream sequence. Further sequence analysis revealed that the cloned DNA immediately downstream of the T7lac sequence from the phagemid is 97% identical to the previously identified *S. epidermidis* second lipase gene, *gehD* (12) (Fig. 2*A*).

Purification and Characterization of Recombinant, Mature GehD—Previous studies of GehD and other staphylococcal lipases have shown that they are transcribed and translocated as 650–700-amino acid precursors that are processed post-translationally to extracellular mature lipases of about 360 amino acids with a size of ~45 kDa (12). To simulate the native protein in the mature form, we used the PCR to construct recombinant mature GehD (Fig. 2*A*). The PCR product encoding mature GehD was cloned into the expression vector pET-Blue-2 (Novagen). The protein was expressed as a C-terminal polyhistidine (His tag) fusion and purified by nickel-chelating chromatography. Mature GehD appears as a single polypeptide at ~45 kDa when analyzed by SDS-PAGE (Fig. 2*B*, *lane 2*).

Primary and Secondary Structure of Mature GehD—Amino acid sequence comparisons did not reveal any significant similarities between the linear amino acid sequences of Cna and mature GehD. Furthermore, the CD spectra of mature GehD shown in Fig. 3*A* is very different from that of Cna (Fig. 3*C*). Deconvolution of the mature GehD data using the SELCON and VARSLC1 programs revealed that the predicted overall secondary structure of mature GehD consists of ~26.5% α-helix, 20.6% β-sheet, and 52.9% coil. This secondary structure composition differs markedly from that of the reported crystal structure of Cna-(151–318): 8% α-helix, 53% β-sheet, and 39% coil (29).

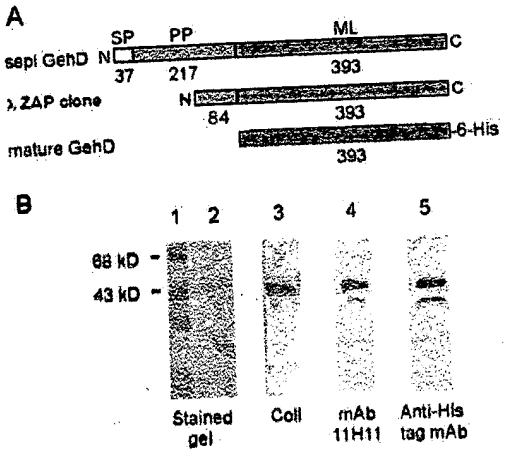

FIG. 2. **Mature GehD binds to collagen.** *A*, schematic representation of the GehD lipase and recombinant constructs. *Sepi GehD*, *S. epidermidis* 9 GehD; λ *ZAP clone*, truncated GehD preproenzyme obtained from phage isolated from the *S. epidermidis* 9491 library; *mature GehD*, recombinant clone created by PCR. The signal peptide (*SP*), propeptide (*PP*), and mature lipase (*ML*) domains are indicated, with their lengths in amino acid residues *below*. *6-His*, six-histidine tag for purification purposes. *B*, recombinant mature GehD was overexpressed and purified using standard techniques. Mature GehD was separated by SDS-PAGE. *Lanes 1* and *2* were stained with Coomassie Brilliant Blue, *lanes 3–5* were transferred to a nitrocellulose membrane and probed with digoxigenin-labeled collagen (*Coll*) or monoclonal antibodies.

In contrast, the mature GehD CD spectra strongly resembles that of a mammalian cell-surface collagen binding receptor known as the α_1 integrin I domain (Fig. 3*B*). The secondary structure composition of this domain is 33.2% α-helix, 20.7% β-sheet, and 46.1% coil, which is comparable with that of mature GehD.

Recombinant Mature GehD Binds to Collagen—The collagen binding activity of the recombinant, mature GehD was analyzed by Western ligand blot. Purified protein was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with digoxigenin-labeled collagen, mAbs 11H11 (anti-Cna), or 7E8 (anti-His tag) (Fig. 2*B*, *lane 4* and *5*, respectively). In this assay, the recombinant, mature GehD binds collagen and both antibodies. It should be noted that a second lower mass polypeptide is detected in *lanes 4* and *5*. This is a contaminating polypeptide that is recognized by the secondary anti-mouse antibody used to detect mAbs11H11 and anti-His.

The collagen binding activity of the recombinant, biotin-labeled mature GehD was also assessed by a solid phase, ELISA-type assay. Mature GehD bound in a concentration-dependent, saturable manner to collagens I, II, and IV coated on microtiter wells (Fig. 4), whereas the binding to wells coated with albumin was minimal. From the ELISA-type assay we estimated that half-maximum binding occurred at about 0.25 μM mature GehD. In addition, we examined the ability of unlabeled mature GehD to inhibit the binding of biotinylated mature GehD to immobilized collagen. Unlabeled GehD could inhibit the binding of the labeled protein to immobilized collagen, whereas a fibrinogen binding recombinant protein from *S. epidermidis* (SdrG) had no inhibitory effect (data not shown). This suggests that both biotin-labeled and unlabeled mature GehD bind with similar affinity to immobilized collagen.

We also tried to characterize the binding of mature GehD to collagen by surface plasmon resonance. In this assay, soluble recombinant mature GehD is run over a sensory chip coated with type I collagen. Using a Hepes-based buffer system, we could calculate a K_D of 4 μM for the interaction. Using a glycine buffer we recorded equilibrium data and calculated a K_D of 3 μM and 1 binding site for mature GehD per collagen monomer. However, not only was the interaction of mature GehD with collagen dependent on the buffer system used, but the collagen binding activity declined as the purified mature GehD was stored for long periods of time. Clearly, these are aspects of the mature GehD binding to collagen that we do not understand at the present, and the K_D values reported above must be taken with caution.

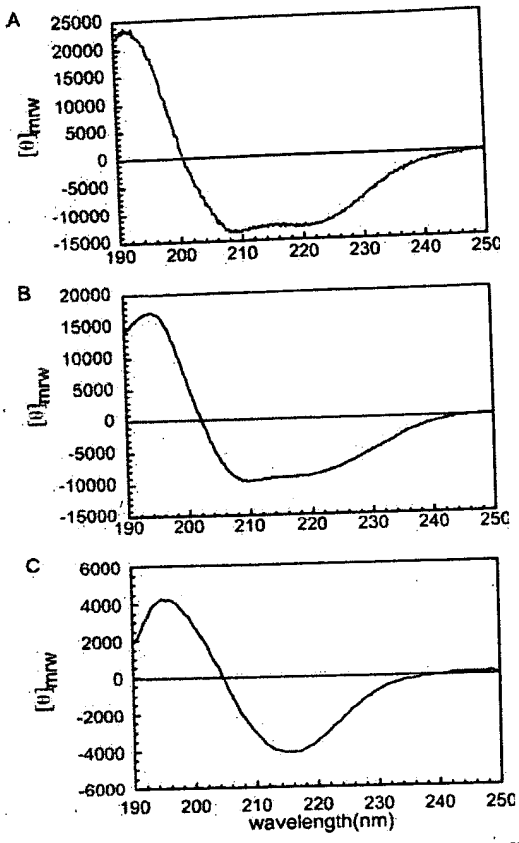

FIG. 3. **Far-UV CD spectra of recombinant, collagen adhesins.** *A*, mature GehD; *B*, α_1 I domain, *C*, Cna-(151–318). The predicted secondary structure composition of each protein is reported under "Discussion." Mean residue weight ellipticity (*θ mrw*) is reported in degrees·cm²/dmol.

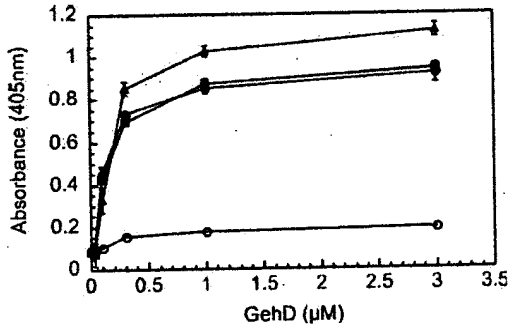

FIG. 4. **Binding of recombinant, mature GehD to immobilized collagens.** Microtiter wells were coated with 1 μg of collagen I (●), II (■), IV(▲), or BSA (○). Increasing concentrations of biotinylated, recombinant GehD were incubated in the wells for 1 h at room temperature. Bound protein was detected with alkaline phosphatase-conjugated streptavidin followed by development with *p*-nitrophenylphosphate substrate. Values represent the means and S.E. of triplicate wells. This experiment was repeated three times with similar results.

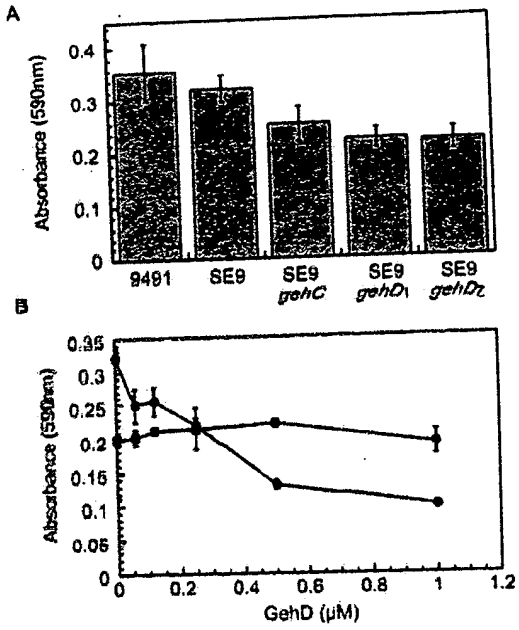

FIG. 5. **Attachment of *S. epidermidis* strains to collagen type I.** *A*, attachment of whole cells onto immobilized collagen. Microtiter wells were coated with 1 μg of type I collagen, washed, and blocked for 1 h at room temperature with BSA. Log-phase *S. epidermidis* 9491, *S. epidermidis* 9, *S. epidermidis* 9 *gehC*::*ermC*, and *S. epidermidis* 9 *gehD*::*ermC* cultures were washed and added to the coated wells. *S. epidermidis* 9 *gehD*$_1$ and *S. epidermidis* 9 *gehD*$_2$ represent two identical, individually isolated clones. Attached cells were detected staining the cells with crystal violet and measuring their absorbance at 590 nm. Values represent the means and S.E. of triplicate wells. This experiment was repeated several times with similar results. *B*, microtiter wells were coated with 1 μg of collagen type I, washed, and preincubated for 1 h at room temperature with increasing concentrations of recombinant, mature GehD. Log-phase *S. epidermidis* 9 (●) and *S. epidermidis* 9 *gehD*::*ermC* (■) cultures were washed and added to the coated wells. Attached cells were detected staining the cells with crystal violet and measuring their absorbance at 590 nm. Values represent the means and S.E. of triplicate wells. This experiment was repeated several times with similar results.

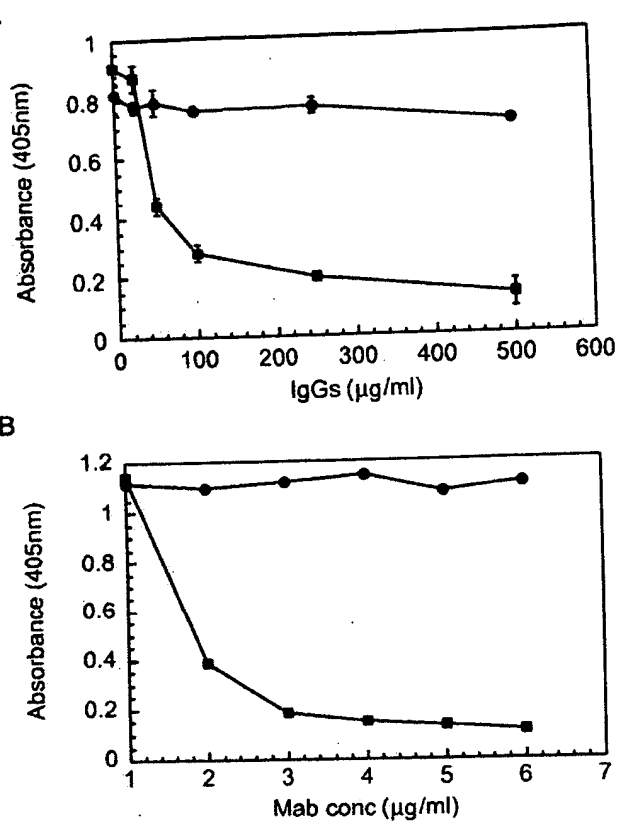

FIG. 6. **Inhibition of mature GehD binding to immobilized collagen.** *A*, recombinant, biotinylated mature GehD was preincubated with anti-GehD (■) or preimmune (●) antibodies before it was added to microtiter wells coated with 1 μg of collagen type I. Biotinylated, bound protein was detected with avidin conjugated to alkaline phosphatase. Values represent the means and S.E. of triplicate wells. *B*, recombinant, biotinylated mature GehD was preincubated with mAb 11H11 (■) (anti-Cna) or mAb 13G12 (●) (anti-FnbpA) antibodies before it was added to microtiter wells coated with 1 μg of collagen type I. Biotinylated, bound protein was detected with avidin conjugated to alkaline phosphatase. Values represent the means and S.E. of triplicate wells.

Purified Mature GehD and Antibodies Can Block the Attachment of S. epidermidis to Collagen—We used a microtiter well attachment assay to study the adherence of *S. epidermidis* to collagen. Two independent, identical clones of *S. epidermidis* carrying a deletion of the *gehD* gene show a decreased ability to attach to immobilized collagen when compared with their isogenic strain, *S. epidermidis* 9. However, the *gehD* mutant strain has a significant residual collagen adherence. A similar strain carrying a deletion in the *gehC* gene has a slight decreased ability to attach to collagen when compared with its isogenic strain (Fig. 5*A*). These data suggests that there may be more than one cell surface adhesin mediating cell attachment to collagen.

The effects of purified, recombinant mature GehD on bacterial adherence were examined in experiments in which collagen-coated microtiter wells were preincubated with increasing concentrations of recombinant mature GehD for 1 h before whole *S. epidermidis* were added. Purified mature GehD inhibited the attachment of *S. epidermidis* 9491 to collagen in a concentration-dependent manner, but it does not affect the already decreased attachment of a *gehD* null strain (Fig. 5*B*). We generated polyclonal antibodies against the recombinant, mature GehD protein and assessed their ability to interfere with the binding of GehD to collagen. Purified anti-mature GehD IgGs effectively inhibit the binding of biotin-labeled mature GehD to immobilized collagen, whereas purified, preimmune IgGs had no noticeable effect (Fig. 6*A*). A similar effect was observed when monoclonal antibodies were used. The monoclonal 11H11 generated against Cna effectively blocked the binding of mature GehD, whereas an unrelated monoclonal, 13G12, did not inhibit (Fig. 6*B*). In addition, we tested the specificity of these antisera using *E. coli* and *S. epidermidis* cell extracts. The anti-mature GehD purified IgGs recognize a polypeptide of ~45 kDa in cell lysates of both *E. coli* expressing the *gehD* gene or *S. epidermidis*. This 45-kDa polypeptide is not present in the *gehD* mutant cell lysates (not shown). These data show that anti-GehD IgGs are specific for mature GehD.

We therefore used these antibodies in a microtiter well attachment assay to test their ability to inhibit the attachment of whole *S. epidermidis* cells to immobilized collagen. *S. epidermidis* cells were preincubated with increasing concentrations of purified anti-mature GehD antibodies before the cell suspensions were added to collagen-coated microtiter wells. Attached cells were detected using crystal violet. Purified, anti-mature GehD antibodies effectively inhibit the attachment of *S. epidermidis* to collagen. Preimmune purified IgGs had no noticeable effect (not shown). The same purified IgGs do not seem to affect the already decreased attachment of the *gehD* null strain (Fig. 7*A*). A similar effect was observed when the monoclonal 11H11 was used to preincubate the bacterial cells (Fig. 7*B*).

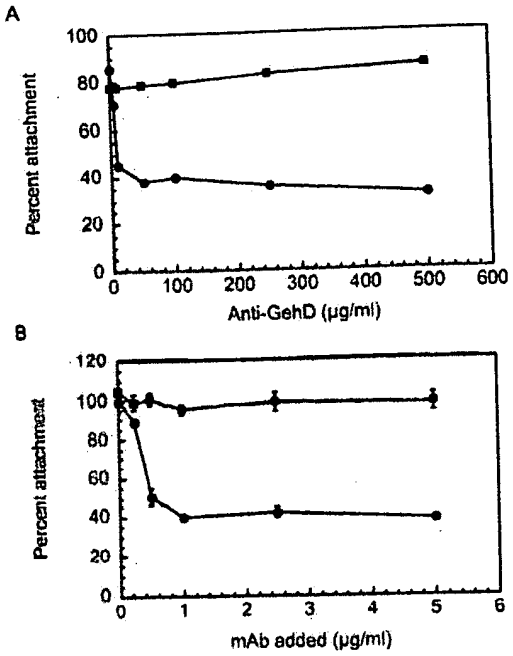

FIG. 7. **Antibodies inhibit the attachment of *S. epidermidis* to collagen type I.** *A*, log-phase *S. epidermidis* 9 (●) and *S. epidermidis* 9 *gehD*::*ermC* (■) cultures were washed and preincubated with anti-mature-GehD before addition to wells coated with type I collagen. Attached cells were detected staining the cells with crystal violet and measuring their absorbance at 590 nm. Preimmune IgGs did not inhibit the attachment (data not shown). Values represent the means and S.E. of triplicate wells. *B*, log-phase *S. epidermidis* 9 (●) and *S. epidermidis* 9 *gehD*::*ermC* (■) cultures were washed and preincubated with mAb 11H11 before addition to wells coated with type I collagen. Attached cells were detected by staining the cells with crystal violet and measuring their absorbance at 590 nm. Monoclonal Ab 13G12 did not inhibit the attachment (data not shown). Values represent the means and S.E. of triplicate wells.

These data suggest that surface-associated mature GehD may act as a collagen adhesin and mediate the attachment of *S. epidermidis* to collagen-coated surfaces.

DISCUSSION

In contrast to *S. aureus*, the adherence of *S. epidermidis* to extracellular matrix proteins has not been well characterized. It is known that *S. epidermidis* can adhere to fibrinogen, fibronectin, laminin (7), and vitronectin (11). The adherence to fibrinogen is mediated by protein adhesins such as Fbe (9) or SdrG (10), and attachment to vitronectin seems to be promoted by the autolysin AtlE. However, the proteins responsible for the interactions with collagen and fibronectin have not been identified. Thus, to search for additional adhesins, we constructed a genomic expression library from the clinical isolate *S. epidermidis* 9491. To screen our library, we took advantage of a panel of 22 mAbs that were raised against Cna-(151–318), the collagen binding MSCRAMM from *S. aureus*. Two of these monoclonals (11H11 and 1F6) cross-reacted to epitopes present on the surface of *S. epidermidis* cells. Therefore, we used mAbs 11H11, 1F6, and labeled collagen to screen our expression library and isolate a collagen binding clone. Surprisingly, the clone that bound to both mAbs and collagen expressed an N-terminal truncation of the GehD preproenzyme. This *S. epidermidis* extracellular lipase has the same overall organization as the other staphylococcal lipases GehC, Geh, *Sal*II, and Lip (22). These lipases appear to be synthesized as preproenzymes consisting of three major domains: signal peptide, propeptide, and mature lipase. The signal peptide is essential for secretion, and it is removed during export of the protein. The propeptide domain has been found to be important for efficient translocation and proteolytic stability during secretion (23). Previous data (12) suggest that GehD is similarly translated as a preproenzyme and post-translationally processed into mature lipase. The size of this active, extracellular lipase is ~45 kDa.

The mature form of GehD can be found associated to whole cells and in lysostaphin extracts from the cell wall.[2] Interestingly, the typical LPXTG motif associated with the cell-wall anchored proteins found in most Gram-positive bacterial surface proteins is not present in the C-terminus of the GehD protein. Recently, several Gram-positive cell-surface adhesins that do not contain a LPXTG motif have been described. These include the fibronectin binding adhesins PavA (24) from *Streptococcus pneumoniae*, FBP54 (25) from *Streptococcus pyogenes*, and the plasminogen binding Eno (26) from *S. pneumoniae*. A possible mechanism for cell surface display of these anchorless adhesins has been described for Eno (26) from *S. pneumoniae*. Eno, a glycolytic cytoplasmic enzyme, is secreted by an unknown mechanism and can re-associate by interacting with receptors on pneumococci. Once Eno is surface-associated, it binds to plasminogen and facilitates the invasion of pneumococci into the host cells. Although the nature of the association between GehD and the *S. epidermidis* cell surface is currently not understood, it is tempting to speculate that, similarly to Eno, it remains associated to the bacterial surface after secretion. Additional proteins with adhesive functions located on the surface without LPXTG motifs include SEN (27), a surface enolase of *S. pyogenes*, SDH (28), a surface dehydrogenase of group A streptococci, and the *S. epidermidis* autolysin AtlE, which specifically binds to vitronectin (11). Anchorless proteins with other biological functions have also been described (29, 30). Clearly, the number of anchorless adhesins identified in Gram-positive bacteria will increase in the future, but it is not clear if these proteins are virulence factors.

The staphylococcal lipases have been considered virulence factors in localized infections such as absesses (13–15), and *in vitro* expression technology (16) showed that lipase gene expression is induced during infection in a murine abscess model. The contribution of these enzymes to virulence is not clearly understood, although lipases may be important for the colonization and persistence of organisms on the skin. Amino acid sequence analysis has shown that GehC and GehD are 51% identical to each other. GehC is closely related to lipase Sal-2 from *S. aureus* NCTC 8530 (84% identity), whereas GehD has greater homologies to the *S. aureus* PS54 lipase, Geh (58% identity), and the lipase of *Staphylococcus hemolyticus*, Lip (70%) identity (12). Although the staphylococcal lipases are a diverse group of enzymes, the predicted secondary structures contain many conserved elements. It would be of great interest to determine whether any of these staphylococcal lipases have adhesive properties in addition to their lipolytic activities. The ability of this enzyme to be bi-functional may be indicative of its importance to the *S. epidermidis* successful colonization and growth on both skin and artificial surfaces.

Mutants of *S. epidermidis* 9 defective in GehD or GehC were used to examine the role of GehD in bacterial interactions with collagen. GehD can mediate bacterial attachment to immobilized collagen. This interaction was blocked by recombinant, mature GehD. In addition, two monoclonal antibodies raised against Cna and antibodies raised against the mature GehD lipase inhibit the attachment of *S. epidermidis* 9 to collagen. Both the *gehC* and *gehD* mutants show a decreased attachment to collagen, which raises the possibility that GehC might also

[2] M. G. Bowden, unpublished information.

interact with collagen. It is interesting to note that we did not find GehC in our library search for collagen adhesins. There are at least two possibilities that could explain this phenomenon; GehC might have a lower binding affinity for collagen, rendering a GehC-expressing clone very hard to detect. Alternatively, when generating the library, the GehC-coding sequence could have been inserted in a different translation frame to that of the vector, thus impeding its correct expression. The ability of recombinant GehC to bind to collagen has not been explored, but it is of future interest.

Mature GehD was identified as a collagen binding adhesin using mAbs raised against Cna-(151–318). However, amino acid sequence comparisons did not reveal any significant similarities between the linear amino acid sequences of Cna and mature GehD. Furthermore, the CD spectra of mature GehD is very different from that of Cna. Deconvolution of the mature GehD data revealed that the predicted overall secondary structure of mature GehD consists of ~26.5% α-helix, 20.6% β-sheet, and 52.9% coil. This secondary structure composition differs markedly from that of the reported crystal structure of Cna-(151–318): 8% α-helix, 53% β-sheet, and 39% coil (31). These data suggest that these proteins may have radically different structures. In contrast, the mature GehD CD spectra strongly resembles that of a mammalian cell surface collagen binding receptor known as the α_1 integrin I domain. The secondary structure composition of this domain is 33.2% α-helix, 20.7% β-sheet, and 46.1% coil, which is comparable with that of mature GehD. We observed several common features between mature GehD and the integrin α_1 I domain. They both bind to collagens, have similar percentage and spatial distribution of α-helices and β-strands, bind divalent cations for full activity, and have open-close conformations (32–35). Because of these common features, it is tempting to speculate that GehD and the integrin α_1 I domain may bind to collagen, adopting similar mechanisms. Although these highly speculative observations may provide some understanding of the collagen binding behavior of GehD, they also underscore the need for a staphylococcal lipase high resolution x-ray structure.

The data described in this work predict that GehD and Cna have radically different secondary structures. However, mAbs 1F6 and 11H11 recognize both proteins. It has been shown that mAb 11H11 recognizes epitopes located in the central segment of Cna-(151–318), and it has been hypothesized that it inhibits ligand binding by directly interfering with collagen within the binding trench (19). This raises the possibility that the collagen binding conformational epitopes present on Cna-(151–318), recognized by 11H11 and 1F6, may also be found in GehD. Therefore, because they seem to recognize a conformational collagen binding epitope, these mAbs can be used as powerful tools to unveil diverse collagen-binding proteins in many other Gram-positive organisms.

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